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Edited by Ramón Latorre, Universidad de Valparaíso, Valparaíso, Chile, and approved November 10, 2008 (received for review July 10, 2008)

Large conductance calcium- and voltage-gated potassium (BK) channels are important regulators of physiological homeostasis and their function is potently modulated by protein kinase A (PKA) phosphorylation. PKA regulates the channel through phosphorylation of residues within the intracellular C terminus of the pore-forming α-subunits. However, the molecular mechanism(s) by which phosphorylation of the α-subunit effects changes in channel activity are unknown. Inhibition of BK channels by PKA depends on phosphorylation of only a single α-subunit in the channel tetramer containing an alternatively spliced insert (STREX) suggesting that phosphorylation results in major conformational rearrangements of the C terminus. Here, we define the mechanism of PKA inhibition of BK channels and demonstrate that this regulation is conditional on the palmitoylation status of the channel. We show that the cytosolic C terminus of the STREX BK channel uniquely interacts with the plasma membrane via palmitoylation of evolutionarily conserved cysteine residues in the STREX insert. PKA phosphorylation of the serine residue immediately upstream of the conserved palmitoylated cysteine residues within STREX dissociates the C terminus from the plasma membrane, inhibiting STREX channel activity. Abolition of STREX palmitoylation by site-directed mutagenesis or pharmacological inhibition of palmitoyl transferases prevents PKA-mediated inhibition of BK channels. Thus, palmitoylation gates BK channel regulation by PKA phosphorylation. Palmitoylation and phosphorylation are both dynamically regulated; thus, cross-talk between these 2 major posttranslational signaling cascades provides a mechanism for conditional regulation of BK channels. Interplay of these distinct signaling cascades has important implications for the dynamic regulation of BK channels and physiological homeostasis.

KCNMA1 | acylation | protein kinase A | maxi-K

Large conductance calcium- and voltage-gated potassium (BK) channels are potently regulated by protein phosphorylation (1) and are important determinants of neuronal, cardiovascular, endocrine, and epithelial function where channel dysfunction may lead to major disorders such as hypertension (2, 3), ataxia (4), epilepsy (5, 6), and incontinence (7). BK channels are potently regulated by phosphorylation, and several putative phosphorylation motifs on the pore-forming α-subunit have been identified (8–12). However, as for other potassium channels, the molecular basis through which phosphorylation of the α-subunit effects changes in BK channel activity is essentially unknown.

BK channel pore-forming α-subunits are encoded by a single gene, *KCNMA1* (13), and native BK channels show functional heterogeneity in their response to protein kinase A (PKA)-mediated phosphorylation. This diversity results, in large part, from the extensive alternative pre-mRNA splicing of the pore-forming α-subunits (10, 12). Previous studies have demonstrated that PKA phosphorylation of a conserved C-terminal phosphorylation motif. $RQPS_{899}$ results in BK channel activation (9, 10, 14). Inclusion of the stress regulated exon (STREX) (15) in the intracellular C terminus generates an additional PKA consensus motif (serine residue 3 of the STREX insert, S3) that results in channel inhibition by PKA (10, 14). PKA inhibition of STREX follows a single-subunit rule, whereby only 1 α-subunit within the BK channel tetramer is required to be phosphorylated by PKA on S3 for inhibition to be conferred (14). Thus, phosphorylation of a single STREX α-subunit probably induces major conformational rearrangements in the BK channel C terminus to mediate channel inhibition.

The STREX insert is cysteine-rich (6 of 58 aa) that, when included into the BK channel α-subunit C terminus, generates a cysteine-rich domain (CRD) in the intracellular linker between the 2 regulator of conductance (RCK) domains (Fig. 1*A*). In many proteins, including other voltage- and ligand-gated ion channels (16–21), cysteine residues are common targets for protein palmitoylation, the covalent attachment of a palmitate lipid to a cysteine residue via a thioester bond. Palmitoylation can exert diverse effects on protein function including allowing cytosolic protein domains to anchor to the plasma membrane (22–24).

We thus hypothesized that palmitoylation of the STREX insert might target the C-terminal domain of the BK channel to the plasma membrane independently of the N-terminal transmembrane domains. Furthermore, this suggested a mechanism by which phosphorylation of a single STREX subunit could result in channel inhibition—through regulation of STREX domain interaction with the plasma membrane. To test these hypotheses, we exploited an integrated imaging, electrophysiological, and biochemical approach. We demonstrate that PKA-inhibition of BK channels results from dissociation of the STREX domain from the plasma membrane. Importantly, palmitoylation of STREX provides a conditional gate for regulation of BK channel activity by PKA phosphorylation.

Results and Discussion

STREX Insert Is a Membrane-Anchoring Domain of the Cytosolic C Terminus of the BK Channel. To address whether the cysteine-rich STREX domain is a membrane-anchoring module we developed an imaging assay to screen the ability of the STREX insert, and its cognate cysteine residues, to anchor the STREX C terminus to the plasma membrane in the absence of the N-terminal transmembrane domains of the α-subunit. We generated fluorescent −GFP fusion constructs of the entire BK channel C terminus (Fig. 1*B*) as well as constructs that encompass the CRD as fluorescent fusions with

Author contributions: L.T., H.-G.K., P.R., and M.J.S. designed research; L.T., O.J., H.M., A.M., I.C.M.R., F.S., and M.J.S. performed research; H.M., L.C., J.G., and L.H.C. contributed new reagents/analytic tools; L.T., O.J., H.M., A.M., I.C.M.R., F.S., and M.J.S. analyzed data; and M.J.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0806700106/DCSupplemental.

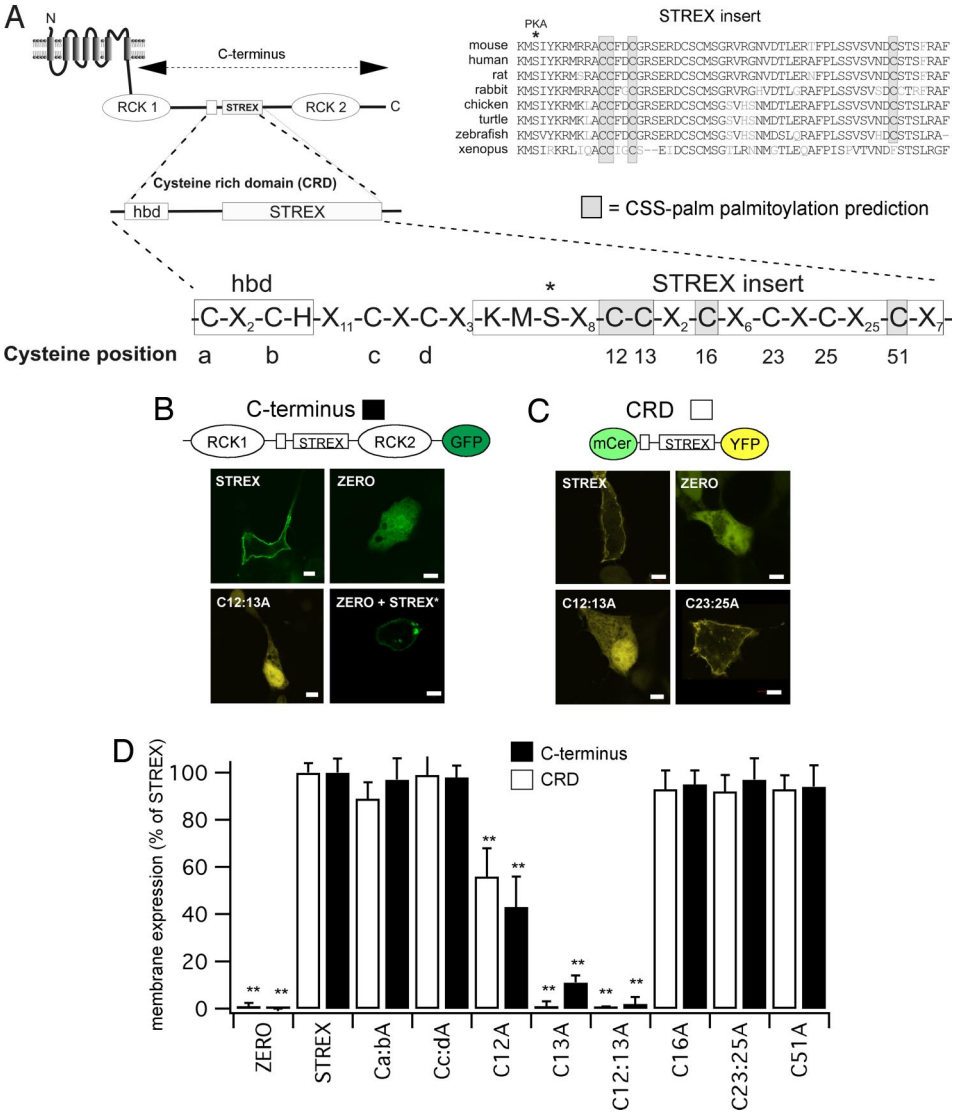

Fig. 1. STREX targets BK channel C terminus to the plasma membrane. (*A*) Schematic illustrating the topology of the BK channel pore forming α-subunit. The STREX insert is located in the linker between the 2 predicted regulator of K^+ conductance (RCK) domains in the intracellular C terminus. Inclusion of STREX generates a CRD encompassing the heme-binding domain (hbd) and STREX. Sequence alignment indicates evolutionarily conserved cysteine residues in the STREX insert predicted to be palmitoylated by the CSS-palm algorithm (shaded) and the PKA phosphorylation site serine S3 (indicated by the asterisk). Cysteine residues are numbered in the CRD as follows: STREX residues numbered from the first STREX residue (K) and upstream cysteines labeled by letters. (*B* and *C*) Schematic of C-terminal GFP fusion (*B*) and CRD domain (*C*) fused between CFP and YFP constructs and representative single confocal section images of STREX, STREX cysteine mutants C12:13A and C23:25A, and the ZERO variant (STREX insert excluded) expression in HEK293 cells. In B *Lower Right*, the C-terminal ZERO–GFP fusion construct was cotransfected with a modified C-terminal STREX construct (STREX*) in which the −GFP tag of STREX was replaced with an −HA epitope. (Scale bars: 5 μm.) (*D*) Summary bar chart of the respective C-terminal (■) or CRD (□) construct localization at the plasma membrane expressed as a percentage of the respective STREX expression. Data are means ± SEM, $N > 12$, $n > 350$ for each construct. **, $P < 0.01$ compared with respective STREX construct (ANOVA with Student–Neuman–Keuls post hoc test).

flanking mCer- and/or −YFP fusion proteins to mimic the STREX linker region between the RCK domains (Fig. 1*C*).

Expression of STREX C-terminal (Fig. 1*B*) or CRD constructs (Fig. 1*C*) resulted in robust plasma membrane expression of the fusion proteins in HEK293 cells in the absence of full-length BK channels or transmembrane segments. Identical results were also obtained in cells that endogenously express STREX variant channels including murine anterior pituitary corticotrope (AtT20) cells, rat pheochromocytoma PC12 cells, and human insulinoma INS-1 cells (data not shown). In contrast, in all these systems, expression of C-terminal, or CRD, fusion proteins that lack the STREX insert (ZERO constructs), but that are otherwise identical to the STREX constructs, did not localize to the membrane (Fig. 1 *B–D*). Coexpression of an −HA-tagged STREX C terminus (STREX*) rescued the ZERO C-terminal −GFP fusion protein localization to the plasma membrane (Fig. 1*B Lower Right*). Thus, a STREX subunit within a heteromeric assembly is sufficient to localize the BK channel C terminus at the plasma membrane. These data demonstrate that STREX acts as a membrane-targeting/anchoring domain and that the key plasma membrane localization motifs must reside within the CRD domain.

To determine whether the cysteine residues within the CRD control membrane localization of the STREX C terminus, we mutated cysteine residues to alanine in both the C-terminal and CRD STREX constructs. Mutation of residue C13 to alanine alone almost abolished membrane targeting in both fusion proteins (Fig. 1*D*), and mutation of its upstream vicinal cysteine residue (C12) significantly reduced membrane localization. The double-mutant C12:13A abolished membrane targeting of both fusion proteins (Fig. 1 *B–D*). In contrast, mutation of any of the other cysteine residues within the CRD had no significant effect on membrane localization (Fig. 1 *B–D*).

Palmitoylation of STREX Is Required for Membrane Targeting of the C Terminus. Using the CSS-palm palmitoylation algorithm (25), we predicted that 4 of the 6 cysteine residues within the STREX insert might be palmitoylated (Fig. 1*A* and Table S1), with cysteine residues 12 and 13 in STREX having the highest CSS-palm scores. Because mutation of C12 and C13 abolished membrane targeting of the STREX C terminus, this data suggests that palmitoylation of these residues controls STREX C terminus association with the plasma membrane. These STREX cysteine residues are highly evolutionarily conserved in vertebrates (Fig. 1*A*).

To directly address whether BK channels are in fact palmitoylated in vivo, we assayed ^{3}H-palmitate incorporation into full-length channels, and the CRD construct, expressed in HEK293 cells. Both full-length ZERO and STREX channels were robustly palmitoylated in HEK293 cells by endogenous palmitoyl transferases (Fig.

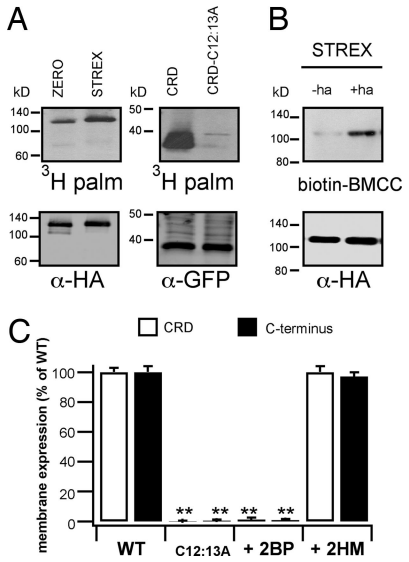

Fig. 2. Palmitoylation of STREX required for membrane localization. (*A*) Representative fluorographs (*Upper*) and Western blots (*Lower*) of full-length STREX-HA and ZERO-HA channels (*Left*) and the wild-type STREX and mutant C12:13A CRD-YFP constructs (*Right*) expressed in HEK293 cells. Constructs were labeled with ^{3}H-palmitate for 4 h and the respective constructs immunoprecipitated (IP) by using α-HA or α-GFP magnetic microbeads respectively and detected by fluorography. (*B*) Representative blots from a cysteine-accessibility assay after IP and treatment of STREX channels with 1 M neutral hydroxylamine to cleave endogenous palmitate thioester bonds to cysteine residues. Accessible cysteines were probed by using biotin-BMCC (*Upper*) with total protein probed by α-HA (*Lower*). (*C*) Summary imaging data of STREX and C12:13A C terminus (■) or CRD (□) as in Fig. 1 *B–D* and the effect of 24-h pretreatment of cells with the palmitoyltransferase inhibitor 2-bromopalmitate (2-BP, 100 μM) or the myristoylation inhibitor 2-hydroxymyristate (2-HM, 0.1–1 mM). Data are means ± SEM, $N > 14$, $n > 950$ for each construct/treatment. **, $P < 0.01$ compared with respective STREX construct (ANOVA with Student–Neuman–Keuls post hoc test).

2*A*). Importantly, the STREX CRD construct is robustly palmitoylated, and ^{3}H-palmitate incorporation was essentially abolished in the C12:13A mutant, suggesting that these residues are the major sites of palmitoylation within the CRD (Fig. 2*A*). As an alternative biochemical approach to confirm endogenous palmitoylation of STREX channels on cysteine residues, we also exploited a cysteine-accessibility assay (26) after treatment of STREX channels with 1 M neutral hydroxylamine (ha). Ha cleaves the thioester bond between endogenous palmitate and cysteine residues, allowing previously palmitoylated cysteine residues to become accessible to the sulfydryl-specific reagent biotin-BMCC (Fig. 2*B*). Functionally, pretreatment of HEK293 cells with the palmitoyl transferase inhibitor 2-bromopalmitate [2-BP (27)] also abolished membrane targeting of the STREX C terminus and CRD fusion proteins in the imaging assays (Fig. 2*C*) as for the palmitoylation-deficient C12:13A mutant. As a control for general effects on posttranslational lipid modification, the myristoylation inhibitor 2-hydroxymyristate had no significant effect on C-terminal or CRD targeting in these assays (Fig. 2*C*), and the vehicle (0.1% DMSO) was also without effect (data not shown). Thus, although the ^{3}H-palmitate-incorporation assays and CSS-palm predictions reveal that cysteine residues common to both the full-length ZERO and STREX channels may be palmitoylated, only the C12 and C13 residues are functionally important for membrane localization of the STREX C terminus at the plasma membrane. Taken together, these data support the hypothesis that the key determinant of STREX domain association with the plasma membrane results from palmitoylation of the STREX insert.

PKA Phosphorylation Dissociates the STREX C Terminus from the Plasma Membrane. PKA inhibition of STREX channels results from phosphorylation of serine residue 3 within the STREX insert (10, 14) that is just upstream of the site of palmitoylation (C12:13). We reasoned that phosphorylation of S3, which would introduce a negative charge into an otherwise basic region immediately upstream of C12:13, might result in destabilization of the STREX domain with the plasma membrane leading to significant structural rearrangements and thus channel inhibition. We took 3 approaches to test this idea. First, we generated the STREX PKA phosphomimetic S3E by mutating STREX S3 to glutamic acid. The S3E mutation abolished STREX C-terminal or CRD construct localization at the plasma membrane (Fig. 3 *A* and *C*). The serine-to-aspartate mutation (S3D, data not shown) also significantly reduced membrane expression, suggesting that both the S3E and S3D mutants largely mimic PKA phosphorylation of S3. In contrast, phosphomimetic mutation of S899 in the downstream C terminus, which is the major site for PKA activation (9, 10, 14), had no significant effect on STREX C terminus association with the plasma membrane (Fig. 3 *A* and *C*). However, the phosphomimetic mutations do not allow us to distinguish between a plasma membrane trafficking defect and a reduced association of the C-terminal domains once at the plasma membrane. Thus, in the second series of experiments, we acutely treated HEK293 cells, expressing the wild-type STREX C-terminal or CRD constructs at the plasma membrane, with the membrane-permeable cAMP analogue 8-CPT-cAMP to establish whether constructs resident at the plasma membrane could be dissociated by PKA phosphorylation. Pretreatment of cells with 0.1 mM 8CPT-cAMP, in the presence or absence of 10 nM okadaic acid, significantly reduced membrane association of the STREX constructs (Fig. 3*B*). Importantly, cAMP-induced dissociation was abolished by pretreatment with the cell-permeable PKA inhibitor H89. In contrast, activation of protein kinase G (PKG) or C (PKC) had no effect on STREX localization at the plasma membrane (Fig. 3*D*). In addition, neither acute (<3% O_2), hypoxia (Fig. 3*D*), which also regulates BK channels through the STREX insert (28), nor ionomycin-induced calcium elevation or KCl-induced depolarization (Fig. S1) had any significant effect on the plasma membrane localization of STREX. These data support a model in which PKA phosphorylation of STREX results in dissociation of the STREX domain from the plasma membrane.

Palmitoylation Gates BK Channel Regulation by PKA. If this model were correct, we would predict that PKA inhibition of STREX BK channels would be lost in channels that are not palmitoylated. To test this hypothesis, we examined the regulation of wild-type STREX channels and the STREX palmitoylation-deficient mutant C12:13A in electrophysiological assays. STREX channels are robustly inhibited by endogenous PKA closely associated with the channel upon exposure of the intracellular face of the patch to cAMP (Fig. 3 *E* and *G*). The effect of cAMP is abolished by the PKA inhibitor PKI_{5-24}, but not by inhibitors of PKG, PKC, or CaMKII (Fig. S2) and is mimicked by application of exogenous catalytic subunit of PKA (PKAc) (Fig. 3*G*). Although full-length C12:13A channels are robustly expressed at the plasma membrane (Fig. 3 *F* and *H*), suggesting the full-length C12:13A mutant is not itself trafficking deficient, cAMP or PKAc fail to inhibit this mutant. Furthermore, the inhibitory effect of cAMP on wild-type STREX channels was abolished in cells pretreated overnight with the inhibitor of palmitoylation, 2-BP (Fig. 3*G*). The C12:13A mutation, and inhibition of STREX channel palmitoylation by 2-BP, which both dissociate the C terminus from the plasma membrane (Fig. 2*C*), resulted in a right shift in the half-maximal voltage for activation (Fig. S3*a*). Reduced basal activity per se is not responsible for the loss of cAMP-mediated inhibition because cAMP potently inhibited STREX channels recorded under conditions to match the shift in $V_{0.5}$ resulting from the C12:13A

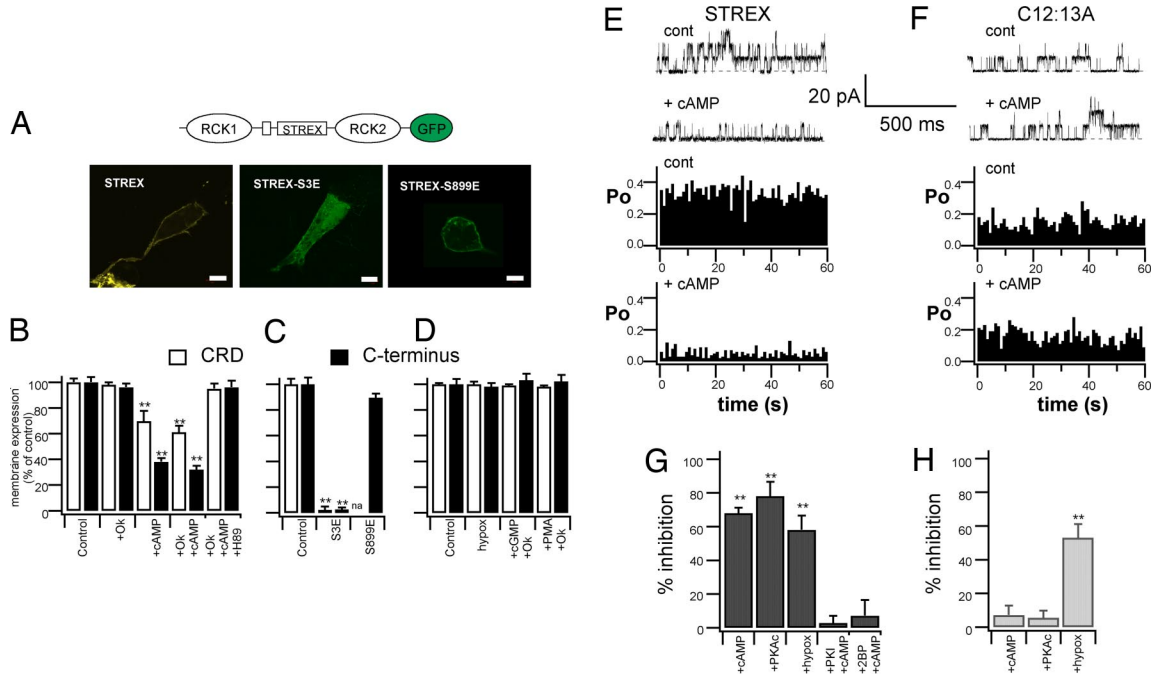

Fig. 3. PKA phosphorylation of STREX dissociates STREX from plasma membrane. (*A*) Representative single confocal sections from HEK293 cells expressing wild-type STREX C-terminal constructs and the corresponding STREX and C-terminal PKA phosphorylation site phosphomimetic constructs S3E and S899E. (Scale bars: 5 μm.) (*B*) Effect of cell-permeable cAMP analogue 8-CPT-cAMP (0.1 mM, 10–30 min) on STREX C terminus (■) or CRD (□) membrane localization in the presence or absence of 10 nM okadaic acid or the PKA inhibitor H89 (1 μM). (*C*) Summary of S3E and S899E construct expression at the plasma membrane (na, S899 site not present in CRD construct). (*D*) Effect of acute hypoxia (<3% O_2), PKG activation with the cell-permeable cGMP analogue 8-CPT-cGMP (0.1 mM in the presence of 10 nM okadaic acid) or PKC activation with the phorbol ester PMA (100 nM in the presence of 10 nM okadaic acid) on construct localization at the plasma membrane. Data are means ± SEM, $N > 4$, $n > 350$. (*E* and *F*) Representative single-channel traces and diary plots of single-channel mean open probability (Po) from isolated inside-out patches of HEK293 cells expressing full-length STREX (*E*) or C12:13A (*F*) channels before and 10 min after exposure to cAMP. Single channels were assayed in physiological K^+ gradients exposed to 0.2 μM free calcium and 2 mM Mg-ATP. (*G* and *H*) Inhibition of STREX (*G*) or C12:13A (*H*) channel Po by cAMP (0.1–1.0 mM) in the presence or absence of the PKA inhibitor PKI_{5-24} (0.45 μM) or 24-h cell pretreatment with 100 μM 2-BP; application of catalytic subunit of PKAc (300 nM) or exposure to acute hypoxia (<3%O_2). Data are means ± SEM, n = 5–14 for each treatment. **, $P < 0.01$ compared with respective control (ANOVA with Student–Neuman–Keuls post hoc test).

mutation (e.g., at −20 mV in 0.1 μM free calcium, inhibition was 67 ± 7% of control, n = 12).

Palmitoylation appears to specifically gate PKA-mediated inhibition of STREX channels because the palmitoylation status of STREX does not alter its intrinsic hypoxia sensitivity (28) (Fig. 3 *G* and *H*) or the regulation by PKG-dependent phosphorylation (Fig. S3*b*). Moreover, inhibition of palmitoylation by 2-BP does not modulate PKA-dependent activation of the ZERO splice variant (Fig. S3*c*) that depends on phosphorylation of a PKA consensus motif (S899) outwith the STREX insert.

Furthermore, the model proposed for palmitoylation-dependent gating of PKA inhibition of STREX would be expected to adhere to a "same-subunit" rule based on the previous demonstration that only a single subunit of STREX needs to be phosphorylated at S3 for channel inhibition (14). The same-subunit model would predict that PKA inhibition, as a result of phosphorylation of STREX at S3, would occur only if the same subunit is also palmitoylated at C12:13. By using a TEA-pore mutation strategy (Y334V) to determine channel subunit stoichiometry (14), cotransfection of subunits that could be palmitoylated but not phosphorylated (constructs with S3A mutation) together with subunits that could be phosphorylated but not palmitoylated (constructs with C12:13A mutation) revealed that cAMP was unable to inhibit channel activity (mean change in activity was 4 ± 10%, n = 12). In contrast, introduction of even a single subunit that could be both phosphorylated and palmitoylated (i.e., a wild-type STREX subunit) with subunits that could be palmitoylated but not phosphorylated (S3A constructs) resulted in robust channel inhibition by cAMP (inhibition was 71 ± 6%, n = 8). These data demonstrate that palmitoylation of the same subunit in which the channel is phosphorylated is required for PKA-mediated inhibition.

To examine whether palmitoylation gates native STREX channel regulation, we examined the regulation of BK channels in mouse anterior pituitary corticotrope (AtT20) cells. STREX variant channels are robustly expressed in this system and are potently inhibited by cAMP-dependent protein phosphorylation (29). cAMP potently inhibited the outward paxilline-sensitive (BK) current in these cells (Fig. 4). Pretreatment of AtT20 cells with 2-BP abolished cAMP-mediated inhibition of the BK current in the whole-cell configuration (Fig. 4). Similar data were obtained in perforated-patch recordings: 8-CPT-cAMP-mediated inhibition of the paxilline-sensitive current in 2-BP-treated cells was only 7 ± 8% (n = 4) of that observed by 8-CPT-cAMP in vehicle-treated controls.

BK channels are remarkable in the range of physiological processes they control, their functional heterogeneity as a result of alternative splicing of the single gene encoding the pore-forming α-subunits, and their extensive regulation by reversible protein phosphorylation. Our data reveal the molecular basis for PKA-mediated inhibition of BK channels through the regulation of STREX domain interaction with the plasma membrane. Palmitoylation of the STREX domain uniquely allows the large intracellular C terminus of the STREX splice variant to associate with the plasma membrane and, importantly, gates the regulation of STREX channels by PKA. Critically, this regulation depends on the site of palmitoylation being adjacent to the site of phosphorylation in the same subunit polypeptide. Importantly, STREX channel activation by PKG-mediated phosphorylation was not affected by palmitoylation status. Furthermore, PKA-activation of ZERO

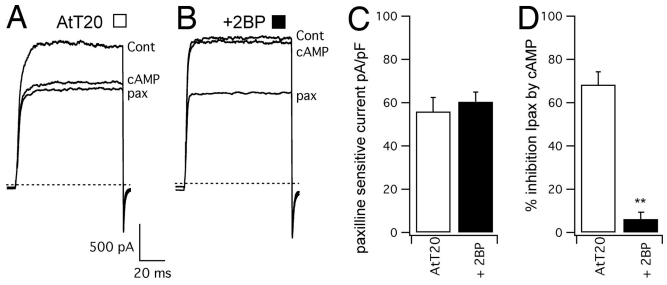

Fig. 4. Palmitoylation gates PKA inhibition of BK channels in native cells. (*A* and *B*) Representative whole-cell outward current traces from mouse anterior pituitary corticotrope (AtT20) cells pretreated with vehicle (0.01% DMSO) (*A*) or 2-BP (24 h, 100 μM) (*B*) before (control) or 10 min after intracellular dialysis with 0.1 mM cAMP or extracellular application of a 1 μM concentration of the BK channel inhibitor paxilline. Cells were voltage clamped at −50 mV in physiological potassium gradients and traces shown recorded during voltage steps to +40 mV for 100 ms. (*C*) Mean current density (pA/pF) of paxilline-sensitive current from vehicle- and 2-BP-treated AtT20 cells determined 90 ms into the pulse at +40 mV. (*D*) Percentage inhibition of paxilline-sensitive current (Ipax) by cAMP in vehicle- and 2-BP-treated cells. Data are means ± SEM, $n = 9$ for each treatment. **, $P < 0.01$ compared with cAMP inhibition in vehicle control (ANOVA with Student–Neuman–Keuls post hoc test).

channels was not affected by inhibitors of protein palmitoylation. PKA and PKG activation of BK channels is independent of the STREX insert and is thought to be mediated by consensus phosphorylation sites in the more distal C terminus (8–10, 12, 14). Importantly, these sites are not juxtaposed to predicted sites of palmitoylation, further supporting our model in which the cross-talk between PKA inhibition and palmitoylation status of STREX depends on both PKA phosphorylation and palmitoylation being in close proximity on the same subunit in the tetramer, thus obeying the single-subunit rule of PKA inhibition (14).

To what extent may such cross-talk between these major, dynamically regulated, lipid and phosphorylation signaling pathways occur in other ion channels? Protein phosphorylation is a fundamental mechanism to control ion channel function, and increasing evidence suggests that multiple ion channels are also regulated by palmitoylation (16–21). We would predict that phosphorylation–palmitoylation cross-talk is likely to occur when sites of palmitoylation are flanked by sites of serine/threonine phosphorylation. In support of this, our pilot sequence analysis reveals (M.J.S. and I.C.M.R., unpublished work) that in several ion channels reported or predicted to be palmitoylated, the sites of palmitoylation are frequently flanked by putative sites for serine/threonine phosphorylation (M.J.S. and I.C.M.R., unpublished work). For example, in ligand-gated GluR6 receptors, the identified sites of palmitoylation are flanked by potential PKC phosphorylation sites, and it has been reported that palmitoylation modifies PKC-mediated phosphorylation of GluR6 receptors (20). Thus, cross-talk between these major posttranslational regulatory pathways, at the level of a single ion channel polypeptide as reported here for BK channels, is likely to be of much broader significance to conditionally regulate a variety of other ion channels.

Although our data reveal that native BK channels are regulated by this palmitoylation–PKA phosphorylation cross-talk, significant challenges for the future will be to examine: (*i*) physiologically relevant signaling pathways that dynamically regulate BK channel palmitoylation and (*ii*) the functional relevance of this cross-talk on cellular and systems physiology. For example, PKA inhibition of BK channels is important for the control of stress hormone secretion in AtT20 pituitary corticotrope cells, and inhibitors of secretion can prevent PKA-mediated regulation of BK channels in this system (29). However, because palmitoylation also controls multiple levels of hormone secretion, including components of the secretory apparatus per se, a major goal for the future will be to develop tools to dissect the relative importance of palmitoylation cross-talk at the level of BK channels compared with other palmitoylation-dependent pathways in native systems.

Palmitoylation, like protein phosphorylation, is a dynamically regulated mechanism (22, 23, 30). Thus, elucidation of signaling pathways that control ion channel palmitoylation, together with examination of cross-talk with phosphorylation signaling pathways as reported here, will provide important insights into the role of these distinct signaling pathways in the control of BK, and other ion channel function and their role in physiological homeostasis.

Materials and Methods

Channel Constructs. The generation of full-length murine ZERO and STREX channel epitope-tagged as well as TEA-pore mutant (Y334V) constructs have been described (14, 31). C-terminal −GFP constructs, the respective CRD constructs, and site-directed mutants were generated and sequence-verified as described in *SI Methods*.

Cell Lines, Transfection, and Treatments. ***HEK293 cells and mouse anterior pituitary corticotrope AtT20 D16:16.*** HEK293 cells and mouse anterior pituitary corticotrope AtT20 D16:16 (passage 18–32) were subcultured essentially as described (10, 14, 29). Cells were transiently transfected by using Lipofectamine 2000 (Invitrogen) or Fugene-HD.

Cell treatments: The palmitoylation inhibitor 2-bromopalmitate (2-BP; Sigma) was made as a fresh 100 mM stock in DMSO and applied at a final concentration of 100 μM overnight. For the myristoylation inhibitor 2-hydroxmyristate (2-HM; Sigma) fresh stock solutions were made either complexed to BSA, or dissolved in DMSO as for 2-BP, and applied at 0.1–1 mM final concentration in each well. For acute imaging assays, cells were incubated for 1 h in DMEM containing 15 mM Hepes and 0.25% BSA (pH 7.4) at 37 °C before incubation for 10–30 min with fresh medium containing the respective drug treatment before rapid fixing. For acute hypoxia, medium was continuously bubbled with nitrogen gas (O_2 level are <3%, equivalent to <25 mmHg) and during fixation.

Imaging Assays. Cells were fixed with 4% paraformaldehyde, mounted in Mowiol, and analyzed under epifluorescence by using an inverted Nikon Eclipse 2000 microscope with a 100× oil objective lens. Confocal images were acquired on a Zeiss LSM510 laser scanning microscope with a 63× oil Plan Apochromat (N.A. = 1.4) objective lens, in multitracking mode to minimize channel cross-talk. Membrane expression was quantified by using the LSM browser or Volocity software as described in *SI Methods*. Data are shown as mean ± SEM for *N* independent experiments where *n* = minimum total number of cells analyzed across experiments for each construct.

Palmitoylation Prediction and Palmitoylation Assays. ***CSS-palm prediction.*** We exploited the published CSS-palm palmitoylation algorithm (25) to predict cysteine residues within the entire coding sequence of the murine STREX BK channel α-subunit as well as the STREX insert alone (see Table S1). Sequences were analyzed with both the published CSS-palm v1.0 algorithm as well as the recently refined CSS-palm v2.0 web interface. In both cases palmitoylation prediction was initially set to the highest cutoff in both algorithms.

^{3}H palmitic acid incorporation. HEK293 cells were transiently transfected in 6-well cluster dishes (≈3 × 10^6 cells per well) with full-length STREX-HA, ZERO-HA channels or the STREX CRD-YFP and CRD-C12:13A-YFP constructs, respectively. Forty-eight hours after transfection, cells were washed, and 1 mL of fresh DMEM containing 10 mg/ml fatty acid-free BSA was added for 30 min at 37 °C. Cells were incubated in DMEM/BSA containing 0.8 mCi/ml ^{3}H-palmitic acid for 4 h at 37 °C, and then the medium containing the free label was removed. Cells were lysed in 150 mM NaCl, 50 mM Tris-Cl, 1% Triton X-100 (pH 8.0), and channel fusion proteins were captured by using magnetic microbeads coupled to HA/GFP antibody (μMACS epitope tag isolation kits, Miltenyi Biotec). After washing columns with 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-Cl (pH 8.0), followed by washes with 50 mM Tris-Cl (pH 7.5) captured proteins were eluted in SDS/PAGE sample buffer [50 mM Tris-Cl (pH 6.8), 5 mM DTT, 1% SDS, 1 mM EDTA, 0.005% bromophenol blue, 10% glycerol] prewarmed to 95 °C. The recovered samples were separated by SDS/PAGE, transferred to nitrocellulose membranes, and probed with either a monoclonal GFP antibody (1:3,000; Clontech) or polyclonal HA antibody (1:1,000; Zymed). A duplicate membrane was dried, sprayed with En^{3}hance fluorographic spray (PerkinElmer–Cetus) and exposed to light-sensitive film at −80 °C by using a Kodak Biomax transcreen LE (Amersham).

Cysteine-accessibility assays. Cysteine-accessibility assays were performed essentially as described by Drisdel (26). Cells were lysed at 4 °C in buffer containing 150

mM NaCl, 50 mM Hepes (pH 7.5), 1.5 mM $MgCl_2$, 1 mM EDTA, and 1% Triton-X-100 containing 25–50 mM *N*-ethylmaleimide (NEM) to block reactive cysteines. Cell lysates were spun, supernatants precleared with protein-G beads (Sigma), and incubated overnight at 4 °C with mouse monoclonal α-HA antibody. Immunopurified channels were rapidly washed 3 times in lysis buffer without NEM and treated with 1 M ha (+ha) (pH 7.4) for 1 h or 1 M Tris·HCl (pH 7.4) as a control (−ha). After washing, beads were exposed to the sulfydryl-specific biotinylating reagent biotin-BMCC (10 μM; Pierce) for 2 h at room temperature. Labeled proteins were run on SDS/PAGE, transferred to PVDF membrane, and probed with streptavidin-conjugated horseradish peroxidase (HRP) and detected by ECL.

Electrophysiological Assays. ***HEK293 cells.*** Single-channel current recordings were performed in the inside-out configuration of the patch-clamp technique, at room temperature (20–24 °C). The pipette solution (extracellular) contained 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 20 mM glucose, 10 mM Hepes (pH 7.4). The bath solution (intracellular) contained 140 mM KCl, 5 mM NaCl, 2 mM $MgCl_2$, 1 mM BAPTA, 30 mM glucose, 10 mM Hepes, 1 mM ATP (pH 7.3) with free calcium $[Ca^{2+}]_i$ buffered to 0.2 μM, unless indicated otherwise. Channel activity was determined during 60-s depolarizations to +40 mV. Data acquisition and voltage protocols were controlled by an Axopatch 200 B amplifier and pCLAMP9 software (Axon Instruments). All recordings were sampled at 10 kHz and filtered at 2 kHz. Channel activity was allowed to stabilize for at least 10 min after patch excision before addition of drugs. Catalytic subunit of PKAc was from Promega. The 8-CPT-cGMP, 8-CPT-cAMP, KT5823, chelerythrine chloride, KN-62, and PKI_{5-24} were from Calbiochem.

Single-channel open probability (P_o) was derived either from single-channel analysis using pSTAT (Axon Instruments) or WINEDR (Version 2.3.9, J. Dempster, University of Strathclyde, U.K.). To determine the mean percentage change in channel activity after a treatment, mean Po or N*Po was measured immediately before and 10 min after the respective drug application. The effect of cAMP and/or PKAc on channel activity was typically maximal by ≈5 min and remained stable over the next 10–30 min after application to inside-out patches. The effect of PKA-mediated phosphorylation was abolished in the absence of Mg-ATP and could be reversed upon application of the catalytic subunit of protein phosphatase 2A (data not shown).

Mouse anterior pituitary corticotrope (AtT20) cells. Whole-cell currents were recording in the conventional whole-cell recording mode of the patch-clamp technique. The bath solution (extracellular) contained 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, and 20 mM glucose (pH 7.4) with or without 0.002 tetrodotoxin. The patch pipette (intracellular) contained 140 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, 30 mM glucose, 1 mM BAPTA, and 1 mM ATP (pH 7.4) with intracellular free calcium ($[Ca^{2+}]_i$) buffered to 200 nM. Perforated-patch recordings were conducted by using amphotericin in the patch pipette. Cells were voltage clamped at −50 mV and depolarized to the respective potentials for 100 ms with leak subtraction applied by using a P/4 protocol and series resistance compensation of >50%. Steady-state outward current was determined 90 ms into the pulse and was stable for ≈30 min under these conditions.

Statistical Analysis. All data are presented as means ± SEM with N = number of independent experiments and n = number of individual cells analyzed in imaging assays. Data were analyzed by ANOVA with post hoc Student–Neuman–Keuls test with significance set at $P < 0.01$.

ACKNOWLEDGMENTS. We thank Dr. Trudi Gillespie of the IMPACT imaging facility in the Centre for Integrative Physiology for assistance in confocal imaging and David L Armstrong for critical reading of earlier versions of the manuscript. James Maurice, Emily Ripley, and Jean-Marc Huibant contributed to pilot analysis of fusion protein localization. O.J. and A.M. were supported by Biotechnology and Biological Sciences Research Council PhD studentships and F.S. by a Medical Research Council PhD studentship. This work was supported by The Wellcome Trust.

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